THESIS

MEASURING THE EFFECTS OF AMMONIA AND DISSOLVED OXYGEN ON JUVENILE BURBOT (*LOTA LOTA*) GROWTH AND SURVIVAL

Submitted by

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ABSTRACT

MEASURING THE EFFECTS OF AMMONIA AND DISSOLVED OXYGEN ON JUVENILE BURBOT (*LOTA LOTA*) GROWTH AND SURVIVAL

Burbot, *Lota lota* are a candidate species for commercial aquaculture because of their palatability and optimal growth at temperatures similar to those used in freshwater trout aquaculture. However, data on burbot environmental tolerances and requirements are sparse, especially with reference to water quality parameters relevant to aquaculture, such as un-ionized ammonia (UIA) and dissolved oxygen concentrations. First, we used a two-phased approach to evaluate the effects of un-ionized ammonia on the growth and survival of burbot. We measured the acute toxicity of ammonia to juvenile burbot (mean SL: 144 ± 6 mm; mean wet weight: 27.3 ± 3.4 g) and calculated a 96-hr LC50 of 0.58 mg·L^{-1} UIA. We then measured the 60-d growth, food consumption rate, and performance of burbot (mean initial SL: 190 ± 6.9 mm; mean initial weight: 67.0 ± 4.5 g) reared in 0.00, 0.03, 0.06, 0.12, or 0.19 mg·L^{-1} UIA using a 20-tank flow-through system under optimal temperature (14.7°C) and dissolved oxygen (DO > 80% saturation) conditions. Elevated ammonia concentration significantly reduced daily food consumption and subsequent growth. Fish exposed to 0.03 and 0.06 mg·L^{-1} UIA showed temporal acclimation to UIA, achieving food consumption and growth rates on par with control fish after 30 days of exposure. The estimated effective UIA concentrations for 10 and 20 percent reductions in growth (EC_{10} and EC_{20}) based on our data are: EC_{10} = 0.03 ± 0.006 mg·L^{-1} and EC_{20} = 0.050 ± 0.004 mg·L^{-1}. We recommend rearing burbot under conditions that keep UIA levels ≤ 0.03 mg·L^{-1} based on our finding that above 0.03 mg·L^{-1} cause measurable reductions in growth rate. Following the ammonia studies, we exposed juvenile burbot (19.5 ± 2.2 g) to five dissolved oxygen concentrations (5.0, 5.8, 6.6, 7.4, and 8.3 (control) mg·L^{-1}) for 9 weeks at 15°C. Variability was high in all treatments, and food consumption and growth rates did not differ among DO levels, although fish at 8.3 mg·L^{-1} grew ca. 29% larger than those at 5.0 mg·L^{-1}. We also measured short-term hypoxia tolerance and resting routine oxygen consumption rates (MO_2) of burbot that had been chronically acclimated to the same DO concentrations. Burbot acclimated to 8.3 mg·L^{-1} lost equilibrium (LOE_{crit}) at a significantly higher concentration (1.85 ± 0.33 mg·L^{-1}) than that of the 7.4 and 6.6 mg·L^{-1} acclimated fish (1.50 ± 0.37 and 1.49 ± 0.27; respectively), while all other groups were intermediate (1.67 ± 0.28). The MO_2s were not statistically distinguishable among acclimation groups (p-value = 0.25), but MO_2 trended lower with decreasing DO concentrations typifying an oxyconforming species. In summary, juvenile burbot are quite tolerant to DO concentrations down to 5.0 mg·L^{-1}. We recommend rearing burbot at DO concentrations > 7.0 mg·L^{-1} and that minimum short-term DO concentrations be kept > 4.0 mg·L^{-1}in culture environments where multiple stressors may be present.

ACKNOWLEDGEMENTS

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CHAPTER 1- THE EFFECTS OF ACUTE AND CHRONIC EXPOSURE OF AMMONIA ON JUVENILE BURBOT (LOTA LOTA) GROWTH AND SURVIVAL

1. Introduction

Fishes in commercial aquaculture facilities are often reared at high densities to maximize economic efficiency, but these conditions can degrade water quality through increased concentrations of ammonia and carbon dioxide, pH fluctuations, and decreasing dissolved oxygen levels (Amend et al., 1982; Colt, 2006; Colt et al., 2009). Ammonia, a nitrogenous waste product of protein digestion, is mainly excreted across the gills of fishes in the form of un-ionized ammonia (UIA) and is toxic to fishes, even at relatively low concentrations (Colt 2006; Kolarevic et al. 2013; Roumieh et al. 2013). For instance, a common recommended upper safe-limit for UIA in salmonid aquaculture is 0.0125 $mg \cdot L^{-1}$ (Timmons and Ebeling, 2010).

Knowledge of how chronic exposure to sub-lethal levels of ammonia impacts fish growth and survival is critical in commercial aquaculture settings where efficient and fast growth is desired, but responses to UIA varies among species. For example, juvenile bighead carp, *Hypophthalmichthys nobilis* showed no growth effects when exposed for 42 days to UIA concentrations up to 0.21 $mg \cdot L^{-1}$ (Sun et al., 2014). In contrast, juvenile Atlantic cod, *Gadus morhua* exposed for 96 days to UIA showed significantly reduced growth at concentrations above 0.06 $mg \cdot L^{-1}$ as compared to a control of less than 0.005 $mg \cdot L^{-1}$ (Foss et al., 2004).

A species ability to adapt to elevated ammonia levels physiologically or behaviorally is linked to its evolutionary history and environment, possibly providing insight to its relative tolerance (Ip et al., 2001; Souza-Bastos et al., 2017). For example, the mountain whitefish *Prosopium williamsoni* is one of the most sensitive fishes to UIA, with a lethal concentration to 50 percent of test animals over 96 hours

(96h-LC50) of 0.14-0.47 mg·L^{-1}; other salmonids of the genus Oncorhynchus are generally considered intolerant as well (Thurston and Meyn, 1984; USEPA (United States Environmental Protection Agency), 2013). These relatively sensitive salmonids occupy high latitude, oligotrophic freshwater systems of North America, Europe, and Asia and share geographic distribution with a freshwater cod, burbot (Lotidae: *Lota lota*) (Crawford and Muir, 2008; Meyer et al., 2009; Stapanian et al., 2010; Vincent et al., 2008).

Burbot are the only freshwater member of the order Gadiformes (cod) and, like their marine relatives, are a palatable food fish (McPhail and Paragamian, 2010). Burbot have a circumpolar distribution, and many populations in Eurasia and North America have experienced declines or extirpations due to habitat loss and over-exploitation (Neufeld et al., 2011; Worthington et al., 2010). These declines and extirpations have triggered attempts to begin stock enhancement efforts through the development of captive culture techniques and the release of hatchery-reared burbot. A spin-off of these conservation and restoration culture efforts is a growing interest in culturing burbot for commercial purposes (Bruce et al., 2020; Kucharczyk et al., 2016).

Studies on the larviculture and reproduction of captive adult burbot have already been completed and have examined the temperature requirements that induce spawning and promote larval survival, with spawning and larvae survival being optimized at around 4°C. (Vught et al., 2007; Zarski et al., 2010). At the beginning of exogenous feeding, larval burbot require small live feed, such as freshwater rotifers before transitioning to larger feeds such as *Artemia* nauplii and ultimately prepared diets (Harzevili et al., 2003; Palińska-Zarska et al., 2014; Vught et al., 2007). Juvenile burbot have shown to grow most efficiently at approximately 2% daily bodyweight rations and prefer marine derived feeds, with an optimal growth temperature at around 15°C (Bruce et al., 2020; Hofmann and Fischer, 2003; Trejchel et al., 2014; Wolnicki et al., 2001). But how juvenile burbot tolerate sub-optimal water quality sometimes encountered in aquaculture facilities, such as elevated UIA, is unknown; information on growth and survival thresholds will aid in the advancement toward efficient commercial production.

Knowledge of their sensitivity to ammonia will be crucial in guiding the culture of this novel species and may help with the protection of wild stocks. The goal of this study was to measure how juvenile burbot tolerate acute and chronic ammonia exposure in a two-experiment approach that was part of a larger multi-institutional study on potential burbot aquaculture. Our first objective was to measure their acute tolerance of UIA following United States Environmental Protection Agency (USEPA) (2013) standard methods for short-term aquatic toxicology studies. The purpose of the acute study was two-fold, first to determine what the acutely lethal level of UIA is at 15°C and, second to use this lethal level to help guide appropriate chronic test concentrations following standard practices (acute-to-chronic ratios). Data on these acute lethal thresholds can help culturists reduce risks of catastrophic fish kills and potentially aid in determining water quality guidelines for natural systems based on the species present. The second objective was to measure how juvenile burbot grow, survive, and perform over a period of 60 days when exposed to sublethal levels of UIA. Because burbot evolved in large cold river and lake systems that are naturally low in total ammonia-nitrogen (TAN), we hypothesized that their ammonia tolerance would be similar to sympatric fishes like the salmonids (Brylińska et al., 2002).

2. Methods and Design

2.1 Origin and husbandry

Juvenile burbot (20 weeks old; 3-7 $g \cdot fish^{-1}$) from the University of Idaho Aquaculture Research Institute (ID) were transported to the Colorado State University Foothills Fisheries Lab (FFL) in Fort Collins (CO). Upon arrival at the FFL, burbot were held in three 850-L circular holding tanks at a biomass density of approximately 6 g per liter. Holding tanks received flow-through (11 $L \cdot min^{-1}$) filtered, aerated, and UV-irradiated surface water from a local lake. Oxygen was supplemented in all three tanks and maintained above 80% saturation using medium pore diffusers. Holding tanks were fitted with covers that occluded 75% of the surface to reduce light levels and additional PVC cover structures were placed inside the tanks (Wocher et al., 2011). Juvenile burbot were hand fed 1.8-mm Skretting Gemma Diamond Cod Feed (57% protein; 15% lipid) pellets at approximately 2% body weight·day $^{-1}$ ration and held at 15°C, which Trejchel et al. (2014) and Wolnicki et al. (2001) reported as the optimal growth temperature for juvenile burbot.

2.2 Acute toxicity (96-h LC50) to unionized ammonia experiments

A 96-hour LC50 experiment was conducted to determine acute toxicity to ammonia following standard methods published by the USEPA. First, a range-finding pilot study was completed to obtain an overview of juvenile burbot sensitivity to ammonia. Target concentrations tested were 0.00, 0.10, 1.00, 2.00, and 4.00 mg·L^{-1} UIA, based on LC50 studies on rainbow trout, Pacific cod, *Gadus macrocephalus* larvae, common carp, *Cyprinus carpio* fingerlings, and the 2013 USEPA Aquatic Life Ambient Water Quality Criteria for Ammonia (Abbas, 2006; Thurston and Russo, 1983; USEPA, 2013; Wang et al., 2015). Juvenile burbot mean weight ± SD was 25.6 ± 2.8 g (n = 10 per tank; age-1) and were held in ten 9-L polyethylene tanks (2 replicate tanks per level) receiving 18 L·h^{-1} of aerated (> 80% saturation) pH-stabilized 15°C water. Temperatures were regulated by mixing heated lake water and ambient temperature lake water with solenoid valves controlled by temperature controllers (Love Series B). Ammonia concentrations in each tank were maintained by variable-output peristaltic pumps (Cole-Parmer Masterflex L/S) that delivered a stock concentration of ammonium chloride (NH_4Cl) at rates that achieved target ammonia levels. Fish were not fed during the 96-hour experiments as per USEPA protocols.

Ammonia concentrations for each tank were recorded at 4-h intervals on day 1 and every 12 hours thereafter. Total ammonia-nitrogen and pH were measured with a digital meter (Orion Star A-324 pH/ISE) and ISE probe (Thermo-Fischer HP9512 for TAN, Thermo Scientific Ross Ultra for pH) to the nearest 0.01 mg·L^{-1} UIA. The digital meter was calibrated daily with fresh standards at temperatures near the experimental water (~15°C). Each tank water sample was pH–adjusted with an ionic strength adjustor (ISA) that raised the pH to above 11 and effectively released all TAN into its un-ionized ammonia (UIA) form. The actual UIA concentrations were then back-calculated using the Thurston et al., (1979) aqueous ammonium equilibrium tables using corresponding TAN, pH, and temperature values. Dissolved oxygen concentrations were recorded with a YSI-200 digital meter and the CSU Soil, Water, and Plant Testing Laboratory tested alkalinity and water hardness using standard EPA methods.

Dead fish were removed immediately upon detection and the time of removal was noted; fish that were moribund (loss of equilibrium and lack of response to gentle prodding) were removed and euthanized in buffered MS-222 (> 250 $mg \cdot L^{-1}$, buffered to a neutral pH with $NaHCO_3$). Time of removal was recorded, and fish were measured (standard and total length in mm, weighed to nearest 0.1 g). At the end of the 96-h period, all surviving fish were kept in the system for an additional seven days while being supplied with ammonia-free water to check for delayed mortality.

A second acute toxicity trial with juvenile burbot mean weight ± SD of 27.3 ± 3.4 g (age-1) was conducted following the range-finding study, testing a more narrow range of concentrations (0.00, 0.10, 0.20, 0.40, and 0.80 $mg \cdot L^{-1}$ UIA). All other components of the second experiment were identical to the range-finding study. LC50 values were calculated using the methods recommended by the USEPA (2002).

2.3 Effects of chronic ammonia exposure

Two hundred randomly selected fish from the holding system were weighed (age-1; 67.0 ± 4.5 g [mean ± SD]) and measured (TL: 221 ± 7 mm [mean ± SD]) before being randomly stocked (10 $fish \cdot tank^{-1}$) into twenty 77-L rectangular aluminum tanks. Tanks represented the experimental units in a single-factor randomized complete block design with five levels of un-ionized ammonia ([control], 0.03, 0.06, 0.12, 0.24 $mg \cdot L^{-1}$) and four replicate tanks per concentration. The UIA concentrations tested were based upon the results of the 96-h LC50 study using common and accepted fish acute-to-chronic ratios (May et al., 2016). All tanks received continuous flows (180 $L \cdot h^{-1}$; >2x tank volume$\cdot hr^{-1}$) of water from a treatment-specific head tank, which kept UIA concentrations constant. The target UIA concentrations were reached by delivering metered amounts of NH_4Cl stock solution to head tanks receiving calibrated flows of aerated water (>80% saturation) at 15°C. Desired concentrations were achieved by the dosing of a stock solution with a peristaltic pump controlled by an ammonium controller (Cole-Parmer Ion Concentration Controller, model IC7685) that continuously monitored the NH_4^+ concentrations in the head tank outflow. UIA was calculated from the ammonium levels, and the in-line ammonium probe was calibrated at least every other day.

In addition to our in-line ammonium probe, we measured ammonia levels via a handheld meter daily in each individual tank to ensure accurate results. Total ammonia nitrogen and pH were measured, and UIA concentrations were determined as described above. Dissolved oxygen, pH, total dissolved solids, and temperature were also measured daily. The experimental well water source was very hard (>500 ppm $CaCO_3$), which can lead to a small but significant underestimation of UIA, therefore we adjusted UIA calculations using the Messer et al., (1984) approach (see Appendix).

Fish were hand fed 3-mm Skretting ONCOR 40 (45% protein; 19% lipid) sinking pellets to satiation twice daily. Laboratory photoperiod was set to the natural photoperiod of Fort Collins, CO (40.585°N) from September through November. Daily feed amounts were calculated to provide the fish with *ad libitum* rations. Excess feed was removed each morning/evening and uneaten pellets were subtracted from offered feed amounts to calculate daily food consumption. Fish were measured on day 30 (period 1), day 45 (period 2), and day 60 (period 3) to calculate tank biomass and intermediate growth rates.

2.4 Chronic toxicity growth parameters

Daily food consumption (% body weight·day^{-1}), specific growth rates (SGR (($\ln W_2 - \ln W_1$) *100)/$days$); where W_2 = final weight of period, W_1 = starting weight of period, $days$ = number of days in period, feed conversion ratios (FCR = grams consumed/grams gained), and relative weights (W_r) were calculated based on total tank biomass. Relative weight was calculated using the Fisher et al., (1996) equation developed for burbot standard weights (W_s), where:

$log10W_s = -4.868 + 2.898\ log10$ total length (mm, burbot > 200 mm). Food consumption (% body weight) was calculated from mean tank biomass of concurrent weigh periods by treatment level to portray changes more accurately.

2.5 Statistical analyses

Normal distribution of data was evaluated using a normal quantile-quantile plot and a Levene's test for unequal variance was performed. One-way ANOVAs were used to test for differences among treatment groups followed by a Tukey's comparison test (JMP®, Version <15.0.0>. SAS Institute Inc., Cary, NC, 1989-2020). A significance level (α) = 0.05 was used for all tests. Effective concentration estimates (EC_x), which compare growth reduction at a UIA concentration to the control level, were calculated with a four-parameter logistic regression model (R^2= 0.96) comparing mean final weights by tank and treatment level. Model fit was determined using AIC and the highest R^2 value. Control fish growth rates were compared to growth rates predicted by the Pääkkönen et al. (2003) Burbot bioenergetic model using Fish Bioenergetics 4.0 (FB4; Deslauriers et al. (2017)) software implemented in program R version 3.5.3 (R Core Team 2019). We used inputs of the known energy density (17,800 joules·gram^{-1}) of the feed and the mean study temperature and fit the model to measured food consumption rates (grams of pellets·day^{-1}).

3. Results

3.1 Acute toxicity (96-h LC50) experiments

Juvenile burbot (TL: 163 ± 5 mm; weight 25.6 ± 2.8 g [mean ± SD]) showed an all-or-none response to the concentrations tested in the range-finding study. Un-ionized ammonia concentrations of 0.79 $mg{\cdot}L^{-1}$ were lethal within the first 6 hours, with fish in the 2.0 and 4.0 $mg{\cdot}L^{-1}$ levels all dying before target concentrations were reached (Table 1.1). All 0.00 and 0.10 $mg{\cdot}L^{-1}$ UIA fish survived, with no delayed mortality observed. The 96-h LC50 estimated by linear interpolation ($y = 0.97 + 0.693x$, where y = % mortality and x = concentration of ammonia) was 0.58 $mg{\cdot}L^{-1}$.

Juvenile burbot (mean TL: 169 ± 7 mm; mean weight 27.3 ± 3.4 g [mean ± SD]) also showed an all-or-none response in the focused range toxicity study (0.00, 0.10, 0.20, 0.40, 0.80 $mg{\cdot}L^{-1}$ target levels (Table 1.1)). All fish exposed to 0.74 (0.80 target) $mg{\cdot}L^{-1}$ UIA died within 6 hours of exposure while all other fish in the experiment survived and experienced no delayed mortality. The estimated 96-h LC50 for un-ionized ammonia based on linear interpolation ($y = -1.334 + 3.155x$) was 0.58 $mg{\cdot}L^{-1}$, matching that determined by the earlier range-finding estimate.

Table 1.1- Mean ± SD water quality parameters measured during the two 96-h LC50 experiments on juvenile burbot, *Lota lota*. Different letters denote a statistically significant difference using a Tukey's HSD comparison test ($\alpha = 0.05$).

Target UIA (mg·L^{-1})	Measured UIA (mg·L^{-1})	Temperature (°C)	pH	Dissolved Oxygen (% saturated)
		Range Finding Experiment		
0.0	0.01 ± 0.01^{a}	15.1 ± 0.01^{a}	8.02 ± 0.04^{a}	89.3 ± 3.00^{b}
0.1	0.10 ± 0.00^{b}	15.1 ± 0.08^{a}	7.99 ± 0.03^{a}	94.6 ± 3.40^{a}
1.0	0.79 ± 0.05^{c}	15.1 ± 0.00^{a}	7.90 ± 0.07^{b}	90.5 ± 9.30^{ab}
2.0	1.21 ± 0.07^{d}	15.1 ± 0.00^{a}	7.78 ± 0.04^{c}	85.6 ± 0.60^{b}
4.0	1.92 ± 0.13^{e}	15.1 ± 0.00^{a}	7.62 ± 0.01^{d}	91.6 ± 3.00^{ab}
		Modified Range Experiment		
0.0	0.00 ± 0.00^{a}	14.8 ± 0.20^{a}	8.15 ± 0.12^{a}	95.9 ± 0.02^{a}
0.1	0.10 ± 0.03^{b}	14.9 ± 0.18^{a}	8.07 ± 0.06^{b}	97.0 ± 0.02^{a}
0.2	0.22 ± 0.04^{c}	14.9 ± 0.19^{a}	8.04 ± 0.07^{b}	94.1 ± 0.03^{ab}
0.4	0.42 ± 0.06^{d}	14.9 ± 0.19^{a}	7.89 ± 0.05^{c}	91.1 ± 0.02^{b}
0.8	0.74 ± 0.17^{e}	14.9 ± 0.11^{a}	7.73 ± 0.04^{d}	88.8 ± 0.02^{b}

3.2 Chronic exposure

Chronic exposure to un-ionized ammonia affected juvenile burbot survival, growth, food consumption, and relative weights over the course of the 60-day growth study (Table 1.2). Survival was uniformly high in all treatments except in the 0.19 mg·L^{-1} UIA (mean measured concentration; target 0.24 mg·L^{-1}), where 25% of the burbot died within the first two weeks of the experiment, which was significantly higher than the combined 1% mortality observed in the other treatments ($p < 0.001$; Table 1.3). The high treatment (0.19 mg·L^{-1}) dosing was terminated after 45 days and excluded from most analyses due to high mortality, low food consumption rates, and welfare concerns about the body condition of the fish, but fish remained in ammonia–free water and all other components of the experiment were continued.

Table 1.2- Sixty-day mean growth, SGR, FCR, and W_r ± SD of juvenile burbot, *Lota lota*. The high (0.19 $mg \cdot L^{-1}$) level data is excluded from this table. Different letters denote a statistically significant difference using Tukey's HSD comparison test ($\alpha = 0.05$). *High FCR and variation reflects low food consumption during the first 30-days and low to no growth during this period.

Treatment UIA concentration ($mg \cdot L^{-1}$)	Growth (g)	SGR (% weight·day^{-1})	FCR (grams consumed: grams gained)	W_r
0.00	62.7 ± 9.8^{a}	1.12 ± 0.12^{a}	0.86 ± 0.03^{a}	86.61 ± 3.27^{a}
0.03	48.2 ± 2.8^{b}	0.90 ± 0.03^{b}	0.85 ± 0.02^{a}	81.03 ± 2.94^{b}
0.06	29.4 ± 4.8^{c}	0.56 ± 0.11^{c}	0.87 ± 0.06^{a}	77.58 ± 1.64^{b}
0.12	2.7 ± 5.7^{d}	0.05 ± 0.13^{d}	$5.13 \pm 8.15^{a*}$	70.30 ± 0.83^{c}

Table 1.3. Mean ± SD water quality parameters of 60-day growth study on juvenile burbot, *Lota lota*. High ammonia (0.191 $mg \cdot L^{-1}$) dosing was stopped on day 45 and corresponding parameter measurements are from days 0-45. Different letters denote a statistically significant difference using Tukey's HSD comparison test ($\alpha = 0.05$), with exception of the survival data that were analyzed with Fishers Exact Test ($\alpha = 0.05$). *Mean UIA following the cessation of ammonia dosing (days 45-60) in the high treatment was 0.005 ± 0.01 $mg \cdot L^{-1}$.

Un-ionized ammonia ($mg \cdot L^{-1}$)*	Total Ammonia Nitrogen ($mg \cdot L^{-1}$)	Temperature (°C)	pH	Dissolved Oxygen (% saturation)	Percent Survival
0.003 ± 0.00^{a}	0.21 ± 0.16^{a}	14.7 ± 0.32^{a}	7.74 ± 0.05^{a}	90.3 ± 3.56^{a}	100^{a}
0.032 ± 0.01^{b}	2.67 ± 0.35^{b}	14.6 ± 0.29^{ab}	7.70 ± 0.06^{b}	86.3 ± 5.14^{c}	95^{a}
0.060 ± 0.01^{c}	5.10 ± 1.11^{c}	14.7 ± 0.33^{ab}	7.73 ± 0.05^{a}	90.4 ± 5.12^{a}	100^{a}
0.121 ± 0.01^{d}	10.87 ± 2.01^{d}	14.7 ± 0.30^{bc}	7.70 ± 0.04^{b}	88.5 ± 4.88^{b}	100^{a}
0.191 ± 0.02^{e}	18.01 ± 6.47^{e}	14.5 ± 0.26^{c}	7.69 ± 0.05^{b}	90.1 ± 4.29^{a}	75^{b}

Final mean wet weights were significantly different among all treatments ($p < 0.001$; Figure 1.1). Mean specific growth rates also varied significantly among treatments during the first period but became more uniform and not significantly different over time between treatments ($p < 0.001$; Figure 1.2). The overall SGR (1-60 days) was significantly different between all treatments

($p < 0.001$; Figure 1.2). Relative weights did not differ among treatments initially but differences among treatments were apparent after 30 and 45 days, with an inverse relationship between relative weight and UIA concentration ($p < 0.001$). Day 60 mean relative weights of the 0.12 mg·L^{-1} treatment were significantly lower than all other levels ($p < 0.001$).

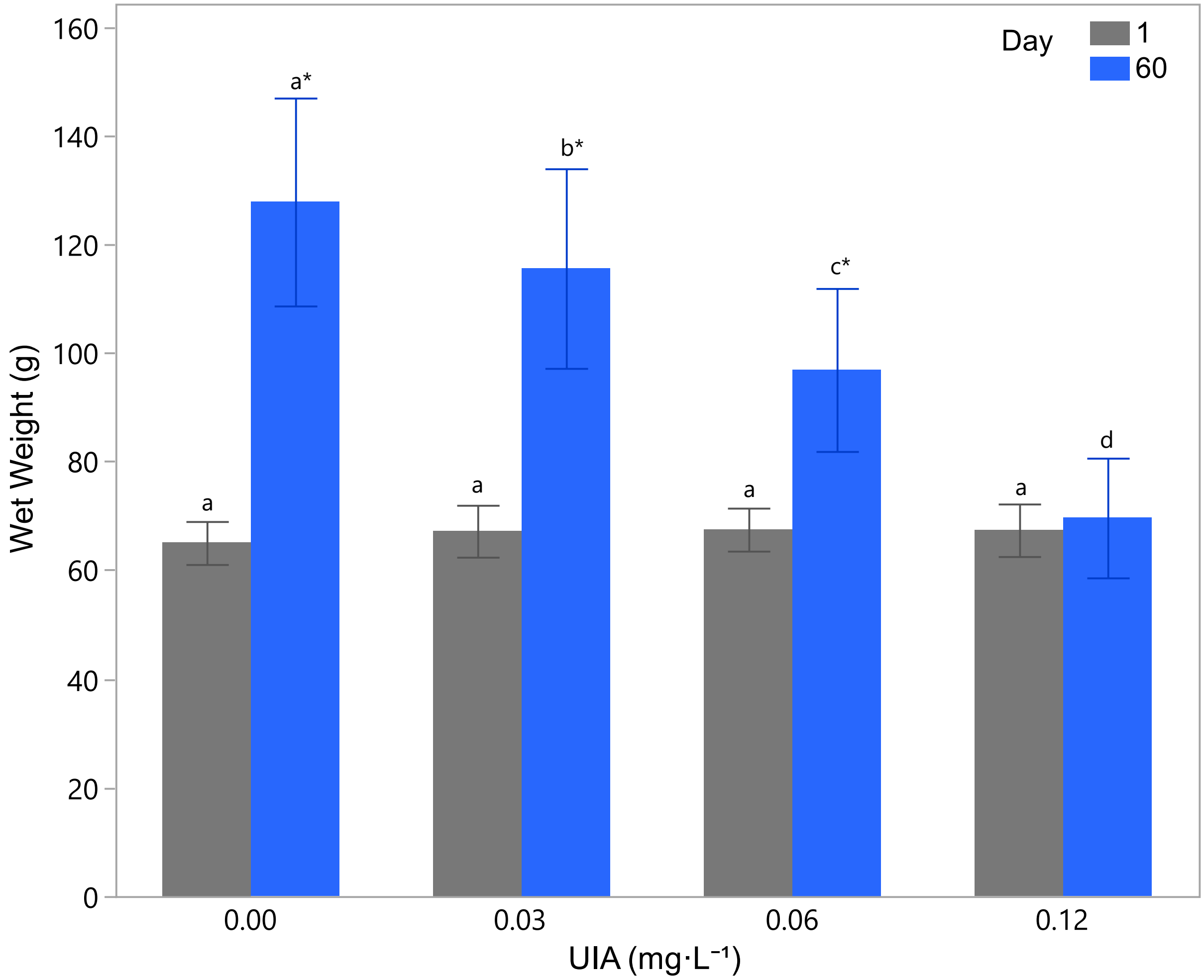

Figure 1.1-Comparison of juvenile burbot, *Lota lota*, wet weights on Days 1 and 60 when chronically exposed to sublethal concentrations of un-ionized ammonia. Different letters denote statistically significant differences between treatment wet weights and asterisks (*) indicate significantly different initial and final wet weights within a treatment using one-way ANOVA and Tukey's comparison of the means test (α=0.05). Values are means ± SD. The 0.19 mg·L^{-1} data are excluded from this figure because the fish were not exposed to 0.19 mg·L^{-1} ammonia for the full 60 days.

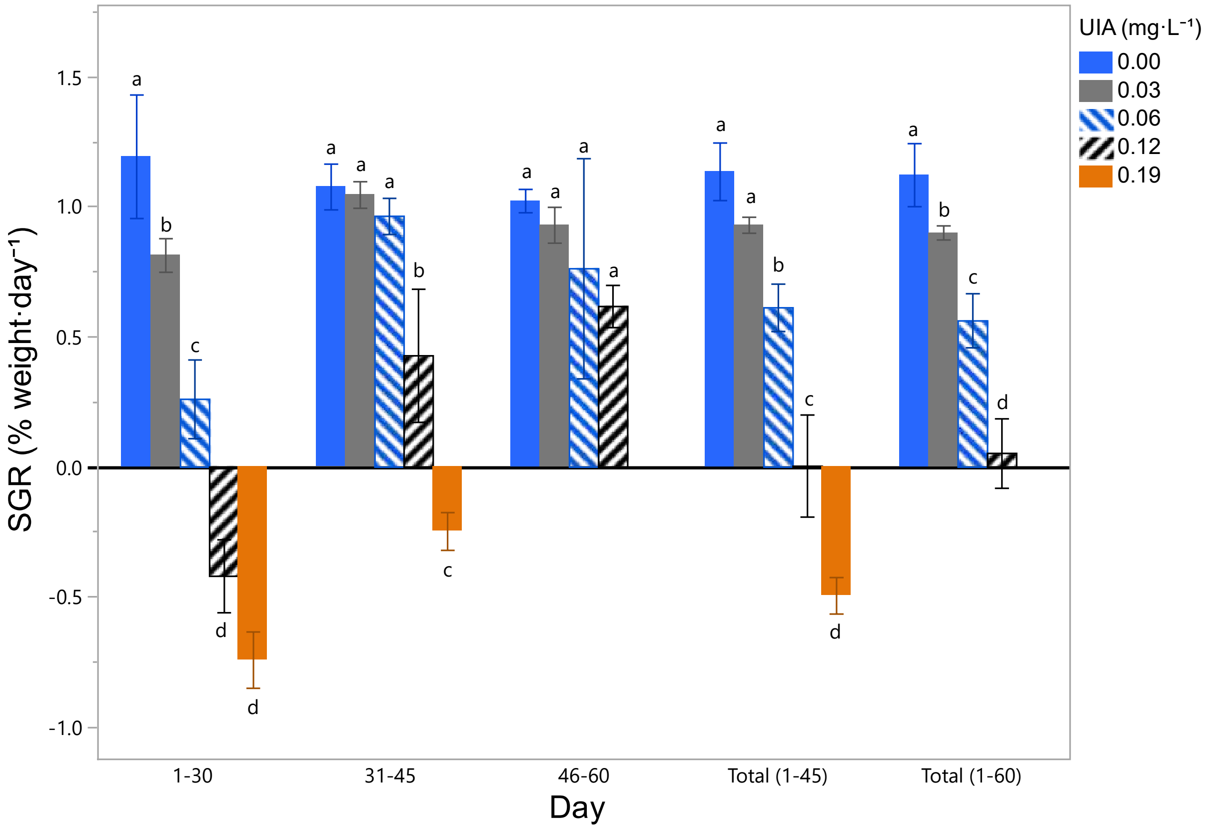

Figure 1.2- Specific growth rates of juvenile burbot, *Lota lota* by time periods separated by weigh days. Different letters indicate statistically significant difference between treatments within a time period using one-way ANOVA and Tukey's comparison of the means test (α=0.05). Bars are mean ± SD. Ammonia dosing of the 0.19 mg·L^{-1} treatment ceased on day 45.

Burbot food consumption rates were significantly affected by chronic exposure to UIA, with consumption rates declining as the exposure level increased over the first 45 days of the study ($p < 0.001$; Figure 1.3). Interestingly, all treatments showed a gradual increase in food consumption rate after the start of the study, with different amounts of time required to reach a potential plateau. Due to low consumption, FCR values were negative for the two highest treatments and were not a good representation of food conversion for period 1. By the final period FCRs were not significantly different ($p = 0.19$).

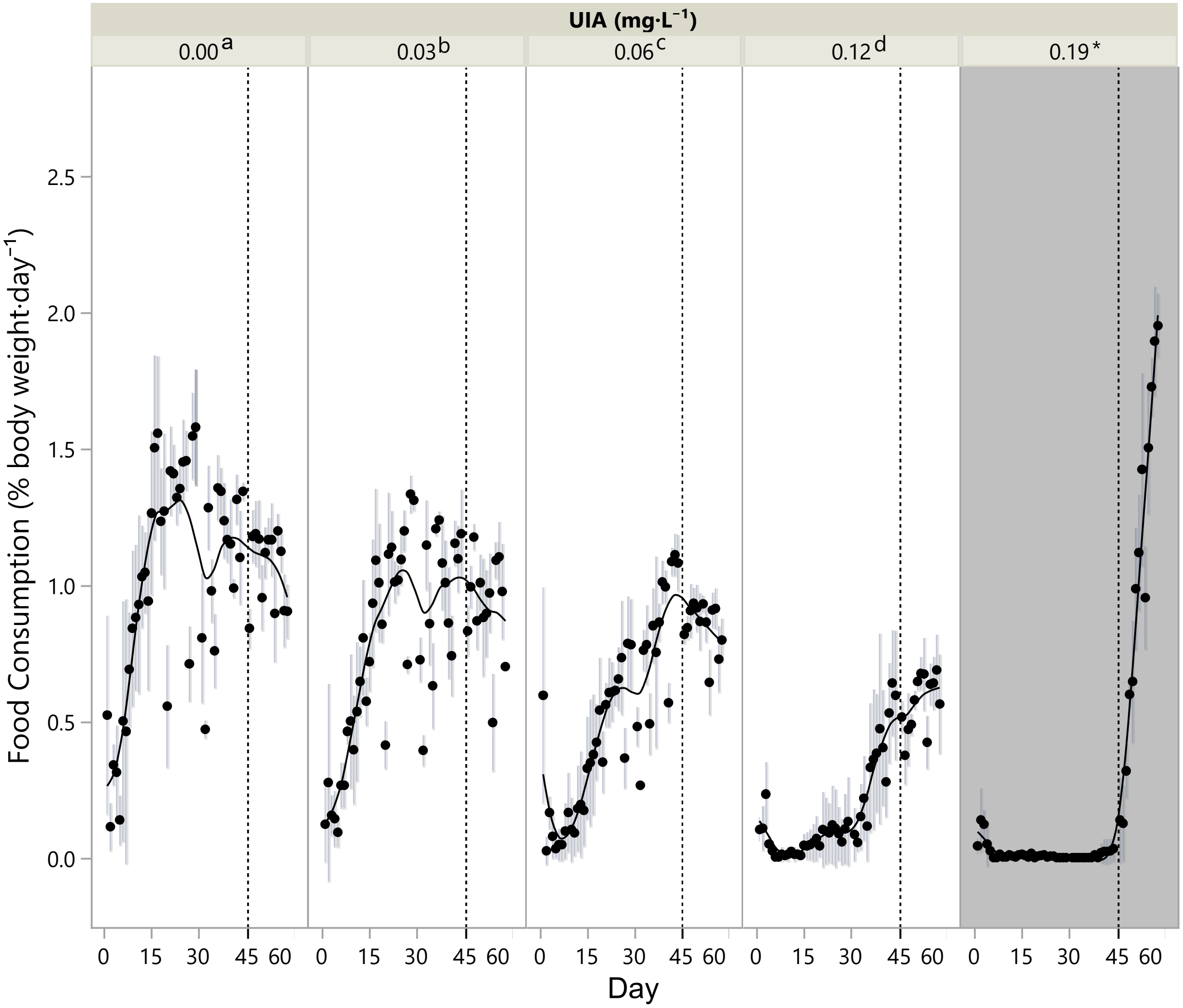

Figure 1.3- Mean daily food consumption rates (percent body weight·day $^{-1}$) by treatment for juvenile burbot, *Lota lota,* exposed to various concentrations of UIA in the 60-day study. Different letters denote significant difference in mean consumption rates using a one-way ANOVA (α=0.05). Error bars ± SD. *The exposure level for the 0.19 mg·L^{-1} treatment was dropped to 0 mg·L^{-1} on day 45.

The effective UIA concentration estimates for 10 and 20% reductions in growth rate compared to the control fish were EC_{10} = 0.03 ± 0.006 mg·L^{-1} and EC_{20} = 0.050 ± 0.004 mg·L^{-1}, respectively. Mean final weights by treatment expressed as a percent of the control mean weight decreased linearly (R^2=

0.94) with increasing UIA. Using actual treatment consumption rates, the FB4 burbot model predictions closely tracked the observed mean control growth (R^2=0.99), but as UIA levels increased, the goodness of the FB4 model fit decreased (Figure 1.4). The high treatment data were excluded from the FB4 model analyses.

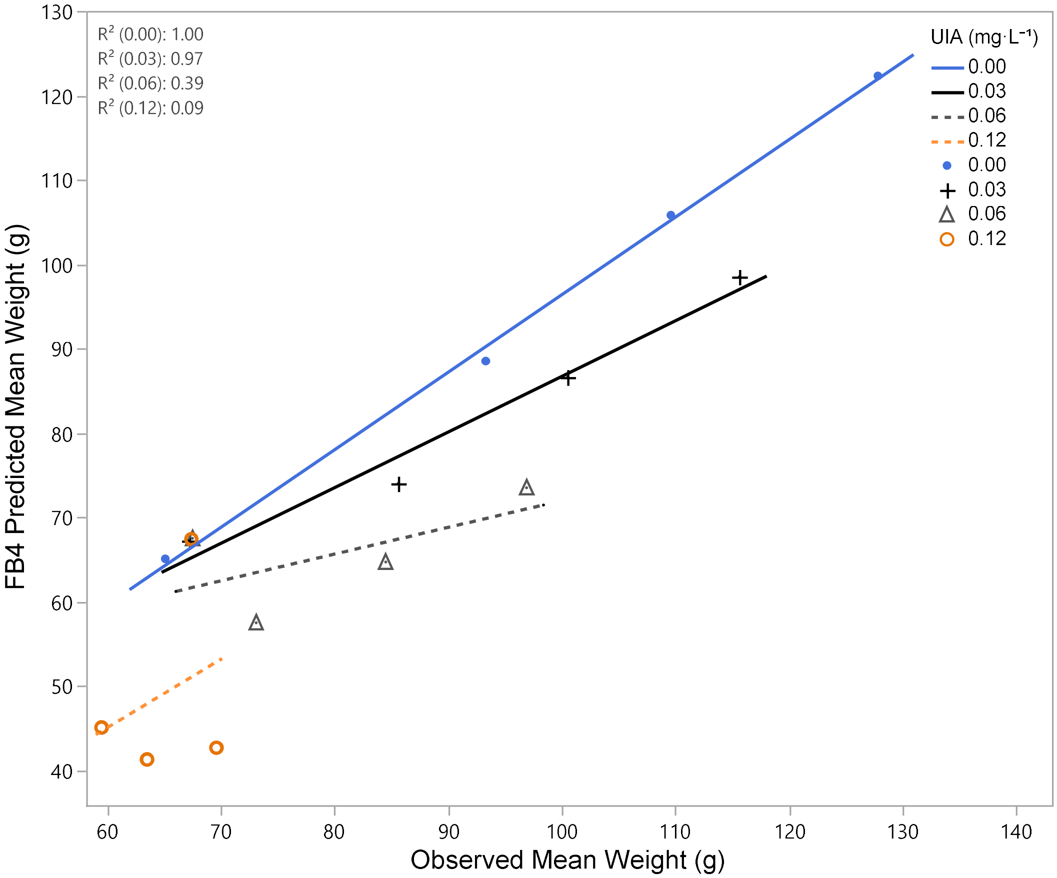

Figure 1.4- Comparison of predicted (Fish Bioenergetics 4.0 model) vs. actual mean weights by treatment level of juvenile burbot, *Lota lota*. Each point is a weigh day mean (n=4). Weigh days occurred on days 1, 30, 45, and 60. The high treatment (0.19 mg·L $^{-1}$) was excluded due to ammonia dosing ceasing on day 45.

4. Discussion

This study was the first to successfully measure the acute tolerance of juvenile burbot to UIA. In addition, we demonstrated that the performance of burbot under aquaculture conditions is inversely related to the sublethal concentrations of UIA present in the water supply (Figure 1.5). The mean specific growth rate for control fish was 1.1 (% body weight·day^{-1}) for this experiment, a higher value than that measured by Wocher et al. (2011) though fish used in this study were smaller (65 g vs. 120 g initial weight) and reared at a warmer temperature (14.7°C vs. 12.3°C). Nevertheless, although conditions such as stocking density, temperature, and fish size were different, our control fish growth rates are indicative of high-quality environmental conditions (temperature, pH, and DO). As environmental conditions were uniform among treatments and dissolved oxygen levels were maintained at ≥ 80% saturation, ammonia level was considered the single factor limiting growth in this experiment.

Figure 1.5- Juvenile burbot, *Lota lota*, final day representatives from UIA chronic exposure growth study.

The all-or-none nature of our acute toxicity test responses precluded the generation of confidence intervals for the estimated LC50 values of 0.58 mg·L^{-1} UIA. As we hypothesized, our results showed that juvenile burbot have an acute ammonia tolerance in the same range as species such as rainbow trout and other salmonids. For example, the juvenile rainbow trout 96-hr LC50 to UIA is 0.58 mg·L^{-1} (TAN = 201.7 mg·L^{-1}, pH = 6.97, temp = 16.6°C) and the juvenile lake trout, *Salvelinus namaycush* 96-hr LC50 is 0.39 mg·L^{-1} (TAN = 83.11 mg·L^{-1}, pH = 7.45, temp = 8.5°C) (Soderberg and Meade, 1993; Wicks and Randall, 2002). Both species occur sympatrically with burbot in high latitude systems that are typically cold and oligotrophic, so it is not unexpected that burbot and other species from these have relatively low ammonia tolerances (Table 1.4). It should be noted that fish exposed in these tests were fasted, which minimizes the effects of UIA as plasma ammonia concentrations are low; a fed or stressed fish would most likely have lower LC50 values (Ip et al., 2001).

Understanding the short-term lethal limits to ammonia could be useful for establishing acute water quality criteria and evaluating potential burbot reintroduction sites. Some aquatic systems that previously supported burbot have been affected by pollution and eutrophication, therefore knowing the acute limits can contribute to assessing the suitability of certain water bodies that are potential candidates for burbot reintroductions (Copp, 1990; Stapanian et al., 2010). From an aquaculture standpoint, acute limits can be used to set never-exceed levels in culture systems. However, under most culture conditions (other than catastrophic system failures) and in most natural systems where burbot still occur, exposure to lower sublethal levels of UIA is the more probable scenario. The subsequent reductions in growth caused by sublethal levels of UIA could mean the difference between profit or loss in a commercial setting or a decline in individual fitness in a natural system.

Table 1.4- Summary of the un-ionized LC50 values for an array of fish species. The relevance column denotes whether the fish are found sympatrically with burbot and/or their relevance to burbot.

Relevance	Species	Size (length/weight)	Temperature (°C)	LC50 (UIA mg·L^{-1})	Source
Aquaculture species	burbot *Lota lota*	27 grams	15	0.58	This Study
	Atlantic salmon Salmo salar	36 grams	8.5	0.61	(Soderberg and Meade, 1993)
	Pacific cod *Gadus macrocephalus*	7.5 mm	10	0.18	(Wang et al., 2015)
	rainbow trout *Oncorhynchus mykiss*	21 grams	9.7	0.5	(Thurston et al., 1981)
	channel catfish *Ictalurus punctatus*	50-76 mm	22	2.4	(Colt and Tchobanoglous, 1976)
Sympatric Species	lake trout *Salvelinus namaycush*	8 grams	8.5	0.39	(Soderberg and Meade, 1993)
	mountain whitefish *Prosopium williamsoni*	57 grams	12.4	0.47	(Thurston and Meyn, 1984)
	shortnose sturgeon *Acipenser brevirostrum*	9.2 grams	17.9	0.58	(Fontenot et al., 1998)
	slimy sculpin *Cottus cognatus*	> 45 mm	10	1.53	(Spencer et al., 2008)

Our study measured the tolerance and effects that elevated UIA has on juvenile burbot growth and survival, but the responses observed may be different in other life stages of burbot. For example, Thurston and Russo (1983) showed a bell-shaped tolerance curve for rainbow trout, with juvenile fish being the most tolerant and in the center of the curve, and larval and adult fish on the edges. Another relevant area of interest is the transition from the larval to juvenile stage in burbot, which is susceptible to high mortality and cannibalism (Jensen et al., 2011). Palińska-Zarska et al. (2014) showed the weaning period (live to pellet feed) is a critical stage with perspective to maximizing survival during this period, and that fish size, rather than age, facilitates higher survival due to the presence of a more developed digestive system when starting burbot on artificial feed. Rearing conditions is another important factor to consider, for example, Wocher et al. (2011) investigated the effects of shelter availability on the growth and activity of juvenile burbot and found no differences in growth between the amount of shelter provided but observed significantly less feeding and swimming activity in fish with higher amounts of shelter. All the above-mentioned factors have the potential to impact how burbot respond to UIA and should be mentioned within the scope of our research.

As seen in other studies, food consumption decreased as UIA concentration increased, and this pattern expressed itself in all our measured growth parameters (Felista Rani et al., 1998; Foss et al., 2004, 2003; Paust et al., 2011; Yang et al., 2011). Interestingly, the results from the burbot in the 0.03, 0.06, and 0.12 $mg{\cdot}L^{-1}$ UIA treatments suggested that fish physiologically acclimated to chronic ammonia levels following prolonged exposure, with the length of the hypothesized acclimation period increasing with UIA concentration. Using a linear interpolation based on the SGR for the final two weeks, we estimated that the 0.12 $mg{\cdot}L^{-1}$ UIA treatment fish would have achieved growth rates on par with the control fish by day 69 ($SGR = -1.35 + 0.03 \times day$). Burbot exposed to the highest UIA concentration probably would not have been able to acclimate to their environment because they consumed almost no food over 45 days of exposure and steadily declined in body condition, possibly revealing a threshold of chronic exposure that could not be compensated for with energetically costly physiological mechanisms. The 0.19 mg·L^{-1} fish displayed poor body condition and would likely have succumbed if the exposure were not ended, as mean W_r on day 45 had declined from a starting value of 77 to a mean of 59 (in contrast, mean W_r on day 45 for control fish had increased to 89). Brown and Murphy (1991) predicted juvenile striped bass, *Morone saxatilis* with a W_r of 63, to have 0% visceral fat, thus indicating extremely poor body condition.

Acclimation to elevated levels of UIA have been observed in Atlantic cod, European seabass, *Dicentrarchus labrax*, bighead carp, and common carp, and increasing rates of ammonia excretion, largely occurring through the gills, is a primary response (Foss et al., 2004; Lemarié et al., 2004; Shrivastava et al., 2016; Sun et al., 2014). We cannot precisely say how burbot acclimated to UIA in this experiment because only growth was measured in this study. However, fish can adjust to high levels of internal ammonia in different ways, including converting ammonia to the less toxic glutamine or increasing excretion rates (Ip et al., 2001; Ip and Chew, 2010). For example, rainbow trout showed significant increases of glutamine levels in the brain as UIA increased, and this is a common nervous system defense against ammonia toxicity (Arillo et al., 1981). As ambient ammonia levels rise, the gradient responsible for UIA diffusion can reverse and cause an influx of UIA into the body. In response, fish can actively pump ammonia out to maintain non-lethal blood ammonia concentrations (Ip et al., 2001). Whether it be conversion to a less toxic substance or actively excreting more ammonia, or both, these adjustments are energetically costly, especially paired with reduced energy intake and thus are plausible explanations for why increasing ammonia concentrations decreased burbot growth in this study.

Further investigations into the rates of burbot ammonia excretion as a function of dietary protein levels is another avenue of research that would provide valuable information that could be used to determine maximal stocking rates as a function of diet and of ammonia tolerance. Prior studies on other species such as the Australian short-finned eel, *Anguilla australis australis* and juvenile silver perch, *Bidyanus bidyanus* have demonstrated that ammonia excretion rates increase with levels of dietary protein, but species-specific information would be warranted (Engin and Carter, 2001; Yang et al., 2002). As is the case for other species, optimizing dietary protein levels for growth while minimizing potential impacts on water quality is an area worthy of further research, especially pertaining to excretion rates related to fish size and life stage (Nowosad et al., 2013; Wright and Fyhn, 2001).

The bioenergetic model developed by Pääkkönen et al. (2003) for wild burbot adequately predicted growth of the experimental control fish, suggesting that this model could be applied to hatchery raised fish. As the ammonia levels rose, the predictions underestimated the observed growth, again highlighting the ability of these burbot to acclimate to increased ammonia over time. The trade-off between rearing fish at higher densities where ammonia concentrations may increase, and the associated reduced growth should be considered by fish culturists, but, along with our data and the Pääkkönen et al. (2003) bioenergetic model, this trade-off can now be quantified in terms of UIA levels. As mentioned before, the threshold density may depend in part upon the dietary protein level of the feed being offered to the burbot, so it may be necessary to conduct further research into ammonia excretion levels as a function of dietary protein inclusion rate.

Our experiment tested only UIA concentrations, but in both aquaculture facilities and natural systems, ammonia may not be the sole stressor. Multiple stressors can combine to have deleterious synergistic effects. For example, Remen et al. (2008) studied the interaction effects of dissolved oxygen saturation and ammonia concentration on the growth of juvenile Atlantic cod and found a significant interactive effect between high ammonia (0.12 $mg \cdot L^{-1}$ UIA) and low dissolved oxygen (68% saturation) on growth rate. As DO levels fall, fish may increase ventilation rate or volume, which paired with elevated ambient ammonia concentrations could increase ammonia exposure and toxicity effects (Foss et al., 2007; Remen et al., 2008). Thus, the results of this study should be taken in context with other relevant water quality issues that may be encountered.

Burbot are an emerging aquaculture species in the United States and Europe and may be an ideal candidate to raise in polyculture with an already well-established species, such as rainbow trout. Polyculture systems can be beneficial in that the producer diversifies their crop and expands their potential market. Burbot have displayed some resistance to common cold water aquaculture diseases caused by *Flavobacterium psychrophilum* and infectious pancreatic necrosis virus (IPNV) and share similar optimal temperature for growth with rainbow trout, consequently the two may fit well together in polyculture systems (Polinski et al., 2010; Windell et al., 1978; Wolnicki et al., 2001). But certainly, more research into disease susceptibility is needed, especially because exposure to reduced water quality such as elevated ammonia concentrations has been linked to increased disease in other species including chinook salmon, *Oncorhynchus tshawytscha* and Nile tilapia, *Oreochromis niloticus* (Abu-Elala et al., 2016; Ackerman et al., 2006)

Based on the results of this study, we recommend that juvenile burbot not be exposed to UIA levels greater than 0.03 mg·L^{-1} for prolonged periods of time. Knowledge of tolerance levels to other forms of nitrogen, such as nitrite, are of equal importance, especially in recirculating systems and is worthy of investigation. In conclusion, our preliminary study of the effects of UIA on juvenile burbot growth and survival should provide a solid foundation for future related research and aid in the advancement of burbot aquaculture.

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CHAPTER 2- GROWTH, METABOLISM, AND DISSOLVED OXYGEN TOLERANCE OF JUVENILE BURBOT

1. Introduction

In commercial aquaculture facilities fish can be reared at densities that create hypoxic conditions, which in turn can decrease growth rates or even result in death (Oldham et al. 2018). Dissolved oxygen concentration (DO) is one of the main limiting factors in determining food intake, growth, and efficient metabolic functioning (Richards 2009; Abdel-Tawwab et al. 2019). Reduced DO can decrease food consumption and consequently overall growth in many fish species, including the channel catfish, *Ictalurus punctatus*, rainbow trout, *Oncorhynchus mykiss*, and Atlantic cod, *Gadus morhua* (Andrews et al. 1973; Pedersen 1987; Chabot and Dutil 1999). For example, Pederson (1987) found the critical DO concentration for growth and food conversion efficiency in rainbow trout reared at 15°C was 7.0 $mg{\cdot}L^{-1}$, with significant decreases in growth at lower concentrations.

As with growth effects, the level of hypoxia that is acutely lethal depends on many factors such as species, size, temperature, and previous hypoxia exposure and acclimation (Nilsson and Östlund-Nilsson 2008; Rogers et al. 2016). Fish can employ a range of physiological and behavioral strategies to cope with hypoxic conditions, including increasing gill surface area or blood hemoglobin O_2 affinity, reducing activity, and increasing gill ventilation rates (Richards 2009; Li et al. 2018). Physiological or behavioral adjustments to low DO may incur an added cost to a fish's energy budget, that in turn can decrease profits for commercial farmers because energy is being diverted from growth to other physiological compartments. Therefore, species-specific knowledge of growth and survival thresholds can aid in improving production efficiency and can also be used to set water quality criteria for controlled and wild situations. One species for which little is known about DO requirements is the burbot (*Lota lota*, Lotidae).

Burbot are the only freshwater representative of the cod Order (Gadiformes) and, like many of their marine relatives, they are demersal predators. Burbot are one of the most widely dispersed freshwater Holarctic fish, occupying the cool and highly oxygenated rivers and lakes of the northern latitudes (Brylińska et al. 2002; McPhail and Paragamian 2010). Unfortunately, in some regions, stocks of native burbot have been depleted or extirpated because of over-harvesting, habitat alterations, and water quality degradation (Copp 1990; Stapanian et al. 2010; Worthington et al. 2010; Bosveld et al. 2015). For example, in the year 2000, the burbot population in the Kootenai River (Idaho, U.S.A.) declined to alarming levels; the projected date of extirpation was estimated to be the year 2015 at the observed rate of decline (Paragamian and Hansen 2011; Hardy and Paragamian 2013). In response, Kootenai Tribe of Idaho (KTOI), in partnership with the University of Idaho, developed techniques for the conservation culture of burbot. These efforts proved successful, and the production techniques are being considered for use in potential commercial aquaculture ventures. The goal of burbot culture for conservation and restoration purposes is to produce suitable numbers of fish that can survive under natural conditions and recruit to the population. Such production is to some degree independent of the cost of production. However, the goal of burbot production in commercial aquaculture is to deliver a niche product to the market in an economically viable manner, so minimizing production costs assumes greater importance.

Burbot are most active at cooler water temperatures, and they spawn during the winter/early spring in northern latitude lakes, rivers, and streams, often under ice cover (McPhail and Paragamian 2010; Zarski et al. 2010). Northern lakes and rivers can experience prolonged periods of hypoxia during winter and spring when ice cover restricts atmospheric gas exchange and photosynthesis is drastically reduced (Leppi et al. 2016; Davis et al. 2019). Although burbot evolved in environments that are generally high in dissolved oxygen, they can encounter hypoxic conditions during times of high oxygen demand (i.e., spawning). Cameron (1973) measured relatively high blood oxygen affinity (P_{50} = 20.5 torr,

P_{CO2} = 3.0 torr at 15 °C) in an Alaskan burbot population, suggesting greater hypoxia tolerance than species like the rainbow trout (P_{50} = 33.1 torr, P_{CO2} = 2.78 torr at 12 °C) (Nikinmaa and Soivio 1979). Other than the Cameron (1973) study, there are no known published data on DO concentration thresholds for growth, general hypoxia tolerance, and hypoxia acclimation effects in juvenile burbot.

Understanding the sensitivity of burbot growth and survival to reduced DO will help culturists determine the optimal and limiting levels of dissolved oxygen concentration for their systems. The first objective of this study was to measure the food consumption and growth rates of juvenile (age-1+) burbot exposed to mild hypoxia over a 9-week experiment at 15°C. The second objective was to quantify hypoxia tolerance by measuring the oxygen concentration that triggered a loss of equilibrium (LOE_{crit}) of burbot acclimated to different levels of hypoxia (Wood 2018). We hypothesized that burbot would tolerate relatively low DO concentrations, given their known P_{50}, and show some acclimation effects because of their ability to be active during periods of potential environmental hypoxia (i.e., in ice-covered lakes and rivers).

2. Methods

2.1 Origin and Husbandry

Juvenile burbot (age 0; 2-5 g per fish) from the University of Idaho Aquaculture Research Institute (Idaho, U.S.A.) were transported to the Colorado State University Foothills Fisheries Lab (FFL) (Colorado, U.S.A.) where they were housed in three 850-L circular holding tanks at a density of approximately 3 g fish per liter. Holding tanks received continuous flows (10 $L \cdot min^{-1}$) of filtered, aerated, and UV-irradiated surface water at 15°C, the optimal growth temperature for juvenile burbot (Wolnicki et al. 2001; Trejchel et al. 2014). Oxygen was maintained above 80% saturation using packed column aerators and supplemental aeration delivered through medium pore diffusers. Holding tanks were fitted with covers that occluded 75% of the surface to reduce light levels, because burbot are cover seeking, especially during daylight hours; additional PVC cover structures were placed inside the tanks (Wocher et al. 2011). The fish were fed a 2% body weight per day ration of 1.8-mm Skretting Gemma Diamond Cod Feed (57% protein; 15% lipid) for 4 months prior to the start of the experiment.

2.2 Effects of chronic hypoxia exposure on growth

Three hundred randomly selected fish from the holding system were measured for wet mass (mean ± SD; 19.5 ± 2.2 g) and TL (mean ± SD; 150 ± 6 mm) before being randomly stocked (15 fish$\cdot$tank^{-1}) into twenty 77-L rectangular aluminum tanks each receiving 144 L$\cdot$hr^{-1} of water for a nine-week growth study (mean initial stocking density = 3.79 kg$\cdot$m^{-3}). Three (200 mm long x 51 mm diameter) PVC pieces provided cover in each tank. Tanks represented the experimental units in a single-factor randomized block design with five nominal dissolved oxygen treatments (8.37 [control], 7.50, 6.70, 5.90, and 5.00 mg$\cdot$L^{-1}) and four replicate tanks per treatment. The DO saturation concentration for the study site (1,564 meters above sea level) was 8.37 mg$\cdot$L^{-1}, and mean measured experimental temperature was 15.2 ± 0.2°C.

Target dissolved oxygen concentrations were reached by mixing 30% air saturated well water with 100% air saturated well water in proportions calculated to achieve the target values. Dissolved oxygen concentrations (mg$\cdot$L^{-1}) were measured (YSI-200 meter) daily in treatment head tanks and individual holding tanks. Total ammonia nitrogen (TAN) and pH were measured weekly using a digital meter (Orion Star A-324 pH/ISE) and ISE probe (Thermo-Fischer HP9512 for TAN, Thermo Scientific Ross Ultra for pH), and un-ionized ammonia concentrations were back-calculated using temperature, pH, and total dissolved solids.

Fish were hand fed *ad libitum* rations of 3-mm Skretting ONCOR 40 (45% protein; 19% lipid) sinking pellets daily, each morning and evening. Feed was evenly distributed throughout the tank to minimize agonistic behaviors. Excess feed was removed each morning/evening and daily food consumption was calculated from known feed amounts. The laboratory photoperiod followed the natural photoperiod of Fort Collins (40.585°N) from May through July. Fish were re-measured at the end of week 3 (period 1), week 6 (period 2), and week 9 (period 3) after anesthetizing them with 25 – 50 $mg \cdot L^{-1}$ of pH-buffered MS-222 to track tank biomass and intermediate growth rates through time. Feed was withheld 24 h prior to weighing.

2.3 Parameters measured and statistical analyses

Daily food consumption (% body $weight \cdot day^{-1}$; F%), and Fulton's condition factor $\left(K = 10^5 \cdot \frac{W}{L^3}\right)$ were calculated using tank biomass during experimental periods. F% was calculated from mean tank biomass of concurrent weigh periods to more accurately portray biomass of tanks as it changed through time. Specific growth rates were calculated following the equation:

$$SGR = \frac{(lnW_2 - lnW_1) \cdot 100}{days}$$

where W_2 = final weight of period, W_1 = starting weight of period, *days* = number of days in period. To investigate tank weight variation, a coefficient of variation for weight (*CV* = (standard deviation/mean tank weight)*100) was used (Jobling 1995). A sample of fish (n = 39) taken prior to the start of the experiment and a final sub-sample of fish (12 randomly selected per treatment) taken at the end of the study were dried for 7 days (to a constant mass) at 60°C to determine percent moisture content and compare percent dry to wet mass ratios (percent dry).

All statistical analyses were conducted in JMP®, Version 15.0.0 SAS Institute Inc., Cary, NC, 1989-2020. Data distributions were evaluated using quantile-quantile plots; Levene's tests for unequal variance were performed. Food consumption differences were tested using a mixed model (repeated measures), with time (days) and DO concentration as fixed effects and tank as a random effect.

All other analyses compared group data during specified experimental periods by one-way ANOVA followed by post hoc Tukey's HSD comparison tests if warranted. A significance level (α) = 0.05 was used for all tests.

2.4 Reduced DO acclimation and $\mathrm{LOE_{crit}}$

A hypoxia tolerance experiment was conducted to test for differences in $\mathrm{LOE_{crit}}$ by acclimation levels using randomly selected fish from the DO groups from the growth study (5.0, 5.8, 6.6, 7.4, 8.3 (control) $\mathrm{mg \cdot L^{-1}}$). Individual oxygen consumption rates were measured using 20, 3.82-L static respirometers following the general design described by Cech (1990) with 20 fish (mean ± SD; wet mass: 45.4 ± 12.7 g) tested per acclimation level over a five day period. Individual fish were loaded 36 hours prior to the start of the experiment to allow time to recover from handling and reach a quiescent state, at which time fish were considered to be at a routine metabolic rate (RMR). Fish were not fed during this period to avoid potential specific dynamic action effects. Respirometers were continuously flushed (18 $\mathrm{L \cdot h^{-1}}$) with water from their respective acclimation group during the resting period. Respirometers were submerged in 77-L water baths to maintain a 15.1 ± 0.2°C (mean ± SD) temperature and were partially covered to reduce outside disturbance; a small observation window in the covers permitted monitoring of the fish to detect LOE. As fish began to show signs of losing equilibrium (e.g. tilting) they were closely monitored until LOE occurred (Chapman et al. 1995).

At the start of the experiment, respirometers were switched from the acclimation level water to ~8 $\mathrm{mg \cdot L^{-1}}$ (near saturation) water for 30 minutes to allow the respirometer DO level to reach saturation. At this point water flow ceased and an initial water sample was taken as per Cech (1990). Temperature and pH were measured at the start of the experiment and DO concentrations were tracked using a Strathkelvin Multi-Channel Oxygen Meter (model 928). Water samples (1 ml) were taken approximately every 40 minutes (mean ± SD; 39 ± 17 minutes) to follow changes in dissolved oxygen concentrations through time. Mass-specific oxygen consumption rate (MO_2; mg O_2 ·kg·hr^{-1}) was calculated following the equation:

$$MO_2 = \left((CO_{2S} - CO_{2E}) \times T^{-1}\right) \times \frac{V}{W}$$

where CO_{2S} = oxygen concentration (mg·L^{-1}) at start of measurement period, CO_{2E} = oxygen concentration (mg·L^{-1}) at end of measurement period, *T* = hours in measurement period, *V* = volume (L) of respirometer, and *W* = mass of fish (kg) (Cech 1990). When LOE was observed, the time was noted, and a final dissolved oxygen measurement was taken. The fish were then measured to nearest 0.1 g wet mass and TL and SL in mm. Respirometer volume was also measured along with final pH and temperature data.

2.5 Reduced DO acclimation and LOE_{crit} analyses

A one-way ANOVA followed by a Tukey's HSD test was used to compare LOE_{crit} values between acclimation groups. Tests of normal distribution and equal variance were conducted as described in the chronic growth study. A mixed model was fit to compare MO_2, with individual fish as a random effect and treatment level and DO concentrations as fixed effects. Oxygen consumption rates were log-transformed to meet the assumption of normal distribution. A significance level (α) = 0.05 was used for all tests.

3. Results

3.1 Chronic hypoxia exposure growth study

Juvenile burbot grew and experienced no mortality under all DO concentrations (Table 2.1). There were no measured treatment effects on growth or condition (Table 2.1). Food consumption rates in all groups initially increased and then decreased throughout the study, but did not differ between groups, although the 7.4 and 8.3 mg·L^{-1} treatments consumed the most on average (Figure 2.1). Peak F% occurred on days 18-19 in all non-control DO groups and on day 39 in the control but were not statistically different from each other during any peak days.

Table 2.1- Measured DO, temperature, survival, growth, and final day condition (*K*) of burbot in a 9-week study. Values are means ± SD. Different letters indicate statistically significant differences between treatments (Tukey's Comparison, $\alpha < 0.05$).

Target Levels (nominal mg·L^{-1})	Measured Dissolved Oxygen Concentration (mg·L^{-1})	Temperature (°C)	Survival (%)	Mass Increase (g)	*K*
8.37	8.25 ± 0.14^{a}	15.3 ± 0.13^{a}	100	11.1 ± 4.3	0.53 ± 0.12
7.50	7.40 ± 0.15^{b}	15.1 ± 0.26^{b}	100	14.2 ± 2.8	0.56 ± 0.08
6.70	6.61 ± 0.15^{c}	15.2 ± 0.32^{a}	100	7.6 ± 3.8	0.51 ± 0.11
5.90	5.80 ± 0.20^{d}	15.1 ± 0.24^{b}	100	10.2 ± 8.1	0.52 ± 0.10
5.00	4.99 ± 0.22^{e}	15.3 ± 0.18^{a}	100	7.9 ± 5.5	0.52 ± 0.10

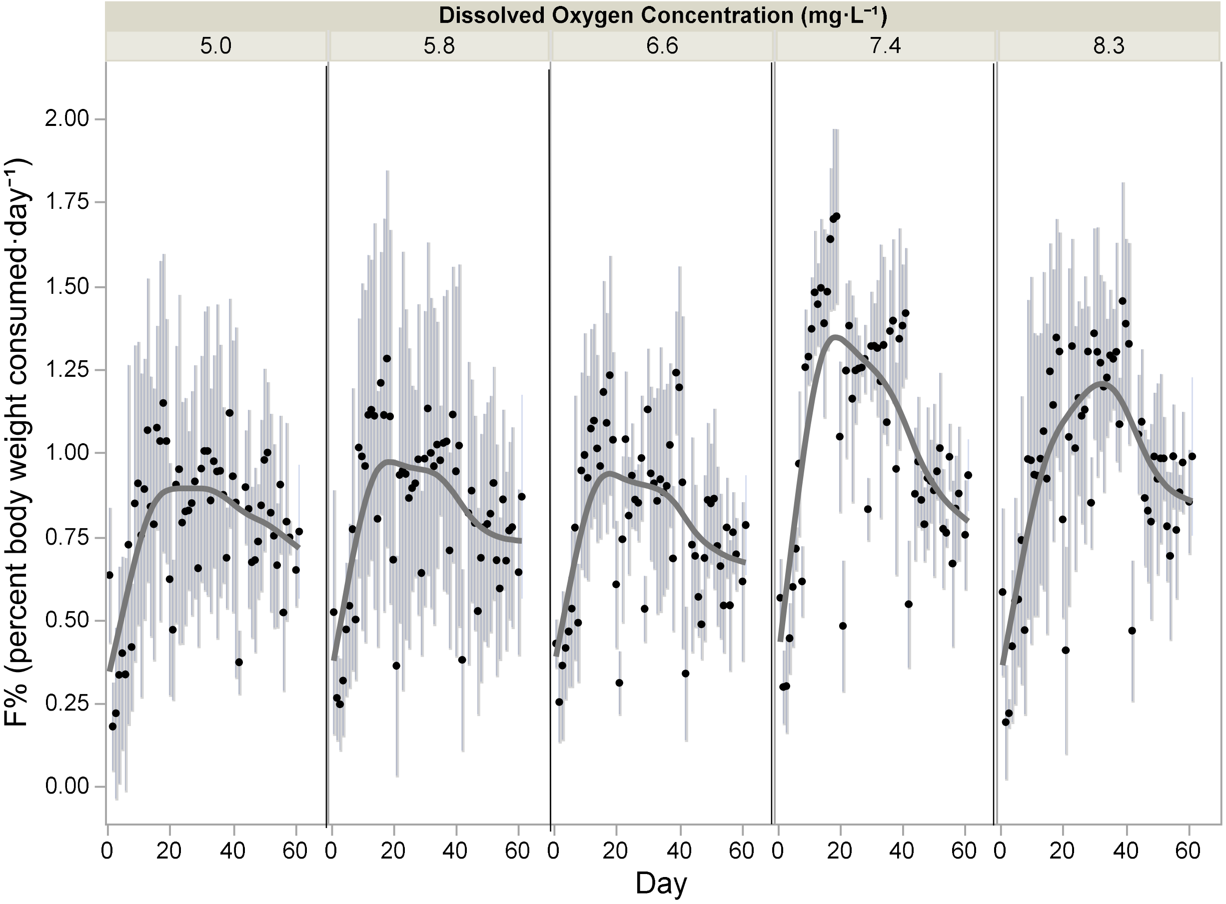

Figure 2.1- Daily mean ± SD, F% of 4 replicate tanks within a DO group (mean measured). Line is a continuous trendline formed by 3rd degree polynomial segments spliced together. There were no statistically significant differences between treatments (Tukey's comparison test; $\alpha = 0.05$).

Final mean masses, although not distinguishable among groups (p-value = 0.46), did significantly increase from initial weights in all groups (Table 2.2). Fish masses within tanks were highly variable in all tanks of all treatments. The overall (9 week combined) coefficient of variation did not differ among treatments (p-value = 0.70) and became more pronounced as the experiment progressed in all groups. Specific growth rates (combined over all 9 weeks) showed some slight differences (p-value = 0.07), with the 7.4 $mg \cdot L^{-1}$ treatment groups displaying the highest overall SGR, followed by the 8.3 $mg \cdot L^{-1}$ fish (Table 2.2).

Table 2.2 Mean ± SD of mass and specific growth rates (% body mass·day^{-1}; SGR) by DO treatment group and time. Period 1 = weeks 1-3, period 2 = weeks 3-6, and period 3 = weeks 6-9.

Time Period

	Initial	*1*		*2*		*3*		*Total (9 weeks)*
Target Levels (nominal mg·L^{-1})	Mass (g)	Mass (g)	SGR	Mass (g)	SGR	Mass (g)	SGR	SGR
8.37	19.3 ± 2.4	21.5 ± 6.6	0.50 ± 0.40	26.9 ± 13.3	1.06 ± 0.26	30.4 ± 18.2	0.59 ± 0.14	0.72 ± 0.23
7.50	19.3 ± 2.3	23.4 ± 5.8	0.97 ± 0.16	29.6 ± 10.6	1.12 ± 0.21	33.5 ± 13.6	0.60 ± 0.14	0.90 ± 0.14
6.70	19.5 ± 2.1	21.4 ± 5.4	0.46 ± 0.35	24.7 ± 10.2	0.68 ± 0.33	27.0 ± 14.0	0.43 ± 0.16	0.52 ± 0.25
5.90	19.7 ± 2.3	22.2 ± 6.1	0.57 ± 0.59	26.6 ± 11.9	0.80 ± 0.53	29.9 ± 16.0	0.53 ± 0.32	0.63 ± 0.46
5.00	19.5 ± 2.1	21.1 ± 5.5	0.39 ± 0.48	24.6 ± 10.7	0.70 ± 0.39	27.4 ± 14.2	0.52 ± 0.14	0.53 ± 0.33

A Games-Howell test estimated significantly higher percent dry weights in the 7.4 mg·L^{-1} treatment compared to the initial reference group and the 5.0 mg·L^{-1} fish (p-value = 0.002 and 0.003 respectively), with no differences in all other comparisons (Table 2.3).

Table 2.3 Mean ± SD, wet, dry, percent dry mass to wet mass, and condition factors (*K*) of initial sub-sample burbot taken prior to the experiment and final treatment group burbot. Different superscript letters denote a significant difference using Games-Howell comparison test (α = 0.05).

Dissolved oxygen group	Wet Mass (g)	Dry Mass (g)	Percent Dry	*K*	N (# of fish)
Initial reference	22.0 ± 2.4^{a}	4.7 ± 0.4^{a}	21.0 ± 1.3^{b}	0.60 ± 0.07^{a}	39
8.25 mg·L^{-1}	29.1 ± 20.2^{a}	7.6 ± 7.7^{a}	24.7 ± 10.6^{ab}	0.50 ± 0.11^{a}	12
7.40 mg·L^{-1}	35.6 ± 16.4^{a}	8.4 ± 4.0^{a}	23.5 ± 1.6^{a}	0.55 ± 0.09^{a}	12
6.61 mg·L^{-1}	30.6 ± 19.5^{a}	6.8 ± 4.8^{a}	21.4 ± 2.8^{ab}	0.52 ± 0.11^{a}	12
5.80 mg·L^{-1}	33.9 ± 20.2^{a}	7.5 ± 5.0^{a}	20.9 ± 2.5^{ab}	0.52 ± 0.09^{a}	12
4.99 mg·L^{-1}	32.6 ± 17.5^{a}	7.1 ± 3.9^{a}	21.5 ± 1.3^{b}	0.55 ± 0.11^{a}	12

3.2 Acclimation and LOEcrit

Burbot in the control group lost equilibrium at a DO concentration of 1.85 ± 0.33 mg·L^{-1}, which was significantly higher than the concentrations that elicited the loss of equilibrium in the 6.60 and 7.40 mg·L^{-1} acclimation groups (p-values = 0.005 and 0.006, respectively). There were no other significant differences among LOE$_{crit}$ values, and no clear trend of tolerance differences resulting from prior acclimation (Table 2.4). Fish masses did not differ between groups (p-value = 0.38; Table 2.4) and were not a significant factor in determining LOE$_{crit}$ (p-value = 0.10). Mean concentration at LOE across all treatments (n = 99 fish) was 1.64 ± 0.34 mg·L^{-1}. Time to LOE (hours) was not impacted by acclimation group (p-value = 0.24) but was significantly influenced by fish wet mass (p-value < 0.001), as larger fish reached LOE faster (*Hours to LOE* = 13.07 – 0.13·*Mass*; R^2= 0.51). Oxygen consumption did not differ by acclimation group (p-value = 0.25), although DO had a significant effect on MO_2 (p-value < 0.001), with overall consumption decreasing as DO declined (Figure 2.2).

Table 2.4- Mean ± SD of weight, time to LOE, concentration at LOE, and partial pressure at LOE of juvenile Burbot following acclimation to reduced levels of dissolved oxygen. Only 19 fish were used for the 6.61 mg·L^{-1} treatment because of one mortality that could not be replaced. *1 fish died during settling period and could not be replaced due to logistical constraints.

Acclimation Level (Mean measured mg·L^{-1})	Mean Weight (g)	Time to LOE (hours)	LOE_{crit} (mg·L^{-1})	LOE_{crit} (% saturation)	N (# of trials)
8.25	49.2 ± 17.9^{a}	6.9 ± 2.7^{a}	1.85 ± 0.33^{a}	22.0 ± 4.0^{a}	20
7.40	46.1 ± 8.2^{a}	6.8 ± 1.6^{a}	1.50 ± 0.37^{b}	17.9 ± 4.5^{b}	20
6.61	43.5 ± 11.5^{a}	7.8 ± 2.0^{a}	1.49 ± 0.27^{b}	17.8 ± 3.2^{b}	19*
5.80	46.5 ± 8.4^{a}	6.7 ± 1.6^{a}	1.68 ± 0.32^{ab}	20.0 ± 3.9^{ab}	20
4.99	41.6 ± 14.7^{a}	8.4 ± 2.8^{a}	1.66 ± 0.26^{ab}	19.8 ± 3.1^{ab}	20

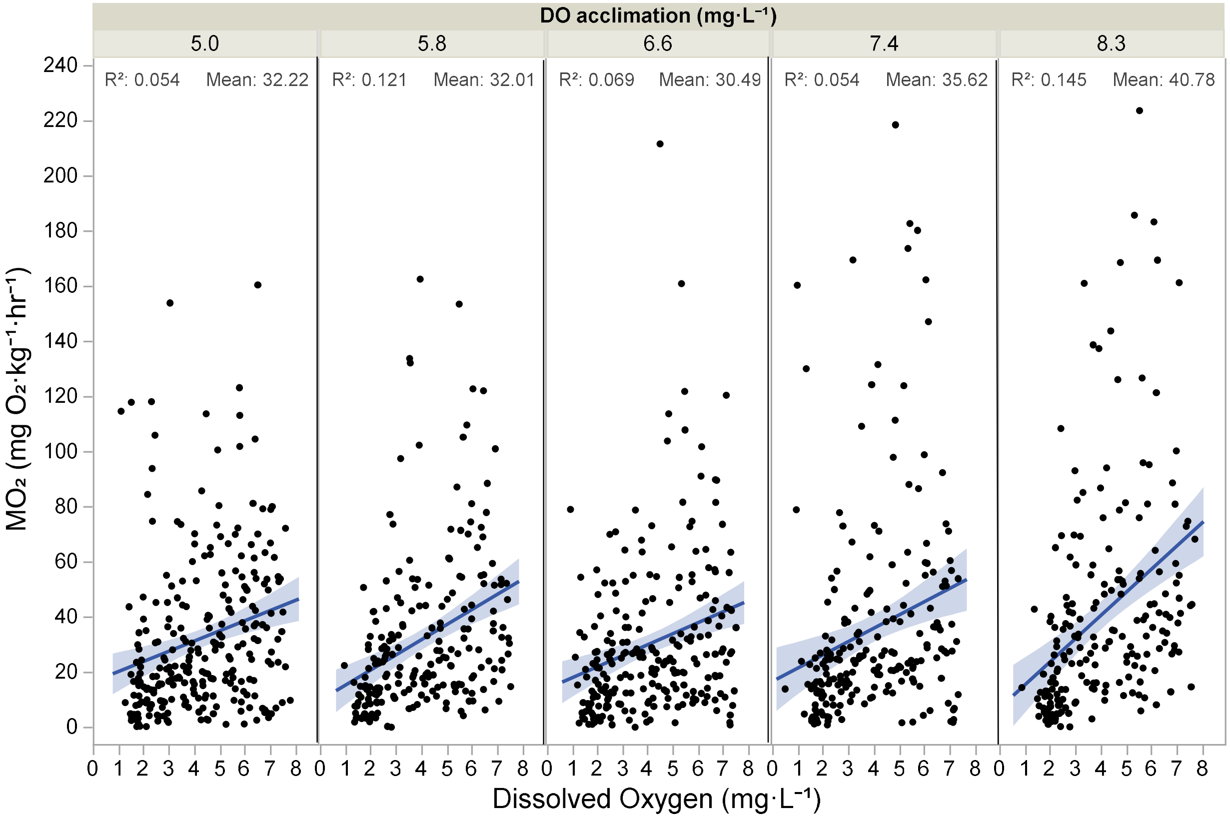

Figure 2.2- Mass-adjusted oxygen consumption (MO_2; mg O_2·kg^{-1}·hr^{-1}) regressed against DO concentration by acclimation levels with linear trendline.

4. Discussion

4.1 Mild hypoxia growth study

To the author's knowledge this study was to the first to measure the effects of DO on the growth and survival of juvenile burbot. Reduced dissolved oxygen concentrations approaching 5 mg·L^{-1} did not significantly influence juvenile burbot growth when compared to higher levels. Although differences were not statistically significant, fish in the 8.3 mg·L^{-1} and 7.4 mg·L^{-1} treatments gained an average of 11.1 and 14.2 g, respectively, which were 29% and 44% higher than the mean weight gain seen in the 5.0 mg·L^{-1} treatment (mean growth = 7.9 g). These hypoxia-related growth reductions were larger than those measured in chinook salmon, *Oncorhynchus tshawytscha,* and sockeye salmon, *O. nerka*, where growth reductions at 5 mg·L^{-1} and 13 °C compared to a 10 mg·L^{-1} control, were 16% and

12%, respectively (USEPA, 1986). Food consumption, *K*, and *SGR* were statistically similar between DO groups, possibly indicating a DO concentration threshold was never reached or other non-treatment factors were involved.

High and temporally increasing CV (%) in fish weight may be indicative of agonistic behavior causing differences in food acquisition or some other mechanism (Jobling 1995; Thorarensen et al. 2015). Jobling (1995) considered poor growth with high weight variation paired with low food conversion rates as a typical sign of social interactions, possibly competition for food or uneven food distribution, and poor growth with little weight variation as an indicator of poor water quality (i.e., DO, ammonia, temperature). In our study, we evenly distributed food across the tank and fed *ad libitum* rations twice daily, such that excess feed was almost always present; thus an uneven distribution and low availability of food were probably not responsible for the high variation in weight. Our water quality variables (other than DO) were always within accepted ranges. Additionally, given that all tanks had large fish, with a mean maximum weight on the final day of 58.7 grams, and all tanks had at least one fish weighing over 40 grams, conditions within tanks were capable of growing large fish. Although dominance behavior was not observed, it is possible a hierarchical positioning occurred that reduced or prevented subordinate feeding. A previous study conducted on juvenile burbot growth under varying ammonia levels in the same experimental system did not show such high growth variation, but these fish were part of a different cohort and initial weights (60 grams) were significantly larger (Vaage and Myrick 2021). Imsland et al. (1998) tested model simulations against laboratory data and concluded that growth variation in juvenile turbot, *Scophthalmus maximus,* was mainly attributed to individual genetic growth factors and their interaction with size hierarchies.

We hypothesize that the heterogenous weights within tanks (Figure 2.3) were attributed to a combination of size-dependent dominance behavior and individual genetic growth differences that were amplified in this highly fecund species (McPhail and Paragamian 2010). Burbot have experienced very little domestication pressure and are therefore still relatively wild, which probably adds to the individual growth variation. Ultimately, a DO threshold that yielded significant reduction in growth was not detected, in large part due to wide variation of weights within tanks, but as Jobling (1995) suggests, there would be little size variation if water quality was poor (low DO) and we observed some individuals in the low DO group with high growth rates, pointing to a non-treatment effect involved in the size gaps.

Figure 2.3- Juvenile burbot from the same tank in the 5.0 mg·L^{-1} treatment at week 9, illustrating the size variation seen in all tanks and DO groups.

4.2 Acclimation effects and LOE$_{crit}$

This study successfully quantified short-term hypoxia tolerance (LOE$_{crit}$) of juvenile burbot, as well as mild hypoxia acclimation effects on LOE$_{crit}$. Mean LOE$_{crit}$ was similar among acclimation groups with the exception of the control burbot, which were significantly more sensitive (LOE$_{crit}$ = 1.85 ± 0.33 mg·L^{-1}). The combined group (without control) LOE$_{crit}$ was 1.58 mg·L^{-1} or 19% DO saturation. Doudoroff and Shumway (1970) summarized acute lethality of many fish species to low DO and found that most experience mortality or loss of equilibrium at concentrations between 1 to 3 mg·L^{-1} (USEPA, 1986). Barnes et al. (2011) reported that Atlantic salmon tested between 14 - 22°C lost equilibrium at 2.19 mg·L^{-1} and Schurmann and Steffensen (1997) measured the critical oxygen saturation for juvenile Atlantic cod at 15°C to be 30.3%, but hypoxia tolerance indices and methods vary, making it hard to compare across species and studies.

One of the most common methods used to evaluate hypoxia tolerance is the determination of a critical point oxygen level (i.e., in kPa, torr, % saturation, mg·L^{-1}) or P_{crit}, which essentially estimates the ambient O_2 level at which a fish transitions from being an oxyregulator to an oxyconformer. Using this method, a lower P_{crit} value indicates more hypoxia tolerance (Urbina et al. 2012; Rogers et al. 2016). Recent discussions have questioned the use of P_{crit} as a good measure of hypoxia tolerance, because not all species exhibit clear oxyregulation, where oxygen consumption rates remain stable until a critical level is reached and MO_2 drops. Estimates of P_{crit} typically use a broken-line regression to determine the DO concentration at which fish switch to oxyconforming, but the MO_2 data for our study did not show a stable rate followed by a steep decline (Wood 2018). In general, burbot MO_2 declined with decreasing DO concentration (Figure 2.2), although in several fish the MO_2 values increased dramatically right before LOE and no piecewise linear trend was visible. Alternative approaches have been proposed, such as LOE_{crit}, and we chose to measure hypoxia tolerance through LOE_{crit}, as we feel it is an ecologically relevant endpoint that can easily be measured in conjunction with P_{crit} if possible (Urbina et al. 2012; Wood 2018).

Our study did not reveal acclimation effects, and other studies have found mixed results with previous hypoxia exposure and subsequent tolerance (Rogers et al. 2016). For example, Remen et al. (2013) measured how Atlantic salmon responded to chronic diel cycling hypoxia exposure (down to 50% saturation) and did not find any acclimation effects on the limiting oxygen saturation, a metric akin to

P_{crit}; similar results have been observed in juvenile snapper, *Pagrus auratus* (Cook et al. 2013). Conversely, qingbo, *Spinibarbus sinensis* showed lower LOE and P_{crit} values after being acclimated to 5 $mg \cdot L^{-1}$ for 30 days, compared to fish reared under normoxic conditions (Dan et al. 2014).

The absence of acclimation effects in our study could be attributed to many factors; test concentrations may not have been low enough to elicit strong physiological responses or the fish may not have spent enough time in the respirometers to adjust, thus negating any previous advantage accrued by the low DO. Mean time to LOE for all treatments was 7.4 hours and mean respirometer volume (mL) to fish mass (g) ratio was 87:1, well above the 30-50:1 recommended ratio of Cech (1990). One common physiological response to hypoxia is a reduction in basal metabolic rate (Li et al. 2018). Mean MO_2 was similar among all treatment groups and differences in MO_2 would have pointed to some level of acclimation but were not detected. Paakkonen and Lyytikainen (2000) measured oxygen consumption rates of burbot fed various rations and measured MO_2 of fasted burbot at 29.5 $mg \cdot kg \cdot hr^{-1}$ which was similar to our overall mean MO_2 of 34.3 $mg \cdot kg \cdot hr^{-1}$.

4.3 Conclusions

Juvenile burbot appear to have intermediate tolerance to hypoxia as growth was not significantly reduced at dissolved oxygen concentrations as low as 5.0 $mg \cdot L^{-1}$ and LOE_{crit} was determined to lie between 1.5- 1.8 $mg \cdot L^{-1}$. However, burbot are more sensitive to low DO than species like the Nile tilapia, *Oreochromis niloticus* (Li et al. 2018). We recommend rearing burbot in at least 7 $mg \cdot L^{-1}$ DO to optimize growth with a minimum of ~4 $mg \cdot L^{-1}$ to avoid high mortality. We hypothesized burbot would be relatively tolerant to hypoxia due their life history, but further research is needed to measure life stage and temperature effects on hypoxia tolerance. Also, low DO combined with other stressors (i.e. ammonia, sub-optimal temperature, hypercapnia, high fish density) are likely scenarios in aquaculture facilities or wild populations that should be explored further, as interactions between stressors can have synergistic effects (Buentello et al. 2000; Foss et al. 2007).

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Appendix

The ionic strength adjustment equation is as follows:

$$\%\, NH_3 = 1/(1 + 10^{(pK_{a,T} - pH - s)})$$

Where $pK_{a,\ T}$= acid dissociation value at temperature (°C) from (Emerson et al., 1975) table 1. *S* incorporates the ionic strength and is calculated as follows:

$$s = \frac{-A\prime I^{0.5}}{1 + I^{0.5}}\,,$$

A' is a coefficient based off the dielectric constant from equation (9) (Messer et al., 1984) and I is the ionic strength based off the total dissolved solids concentration from equation (12) (Messer et al., 1984).